

Molecular diversity in *Lactuca* species using Isozymes and RAPD Markers

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Abstract

Understanding of the molecular basis of genetic diversity in *Lactuca* accessions is substantial for the management, improvement and efficient uses of *Lactuca* accessions. Therefore, this work aimed to evaluate molecular diversity among twenty-six accessions of *Lactuca* species using isozymes and RAPD analyses. The polymorphic percentages were 87.09% and 100% in isozymes and RAPD analyses respectively, indicating a high genetic variation within and among *Lactuca* species. The number of alleles were higher in the wild species compared to the cultivated species, reflecting a reduction in the richness of alleles in the cultivated species due to domestication that caused a reduction in genetic diversity to meet the demand for high crop productivity. Isozymes and RAPD clustering dendrograms: (1) separated, *L. sativa* accessions in more than one cluster confirming their polyphyletic origin; (2) collected the accessions of *L. viminea* in one cluster revealed its homogeneity; and (3) divided the accessions of *L. saligna* in two clusters varied in the number of alleles, particularly "A" form. The corresponding analysis associated the accessions of the wild species based on the alleles "B" of the tested isozymes and the cultivated species on alleles "A" and "C", suggesting that: (1) allele "B" might be the primitive form of these loci that can tolerate the environmental stresses which prevails in the habitats of the wild species, and (2) "A" and "C" could be the derived forms. These results are of great interest for the management of *Lactuca* germplasm and for future breeding programs of lettuce.

Introduction

The genus *Lactuca* L. is a member of subtribe Lactucinae, tribe Lactuceae, that belongs to family Lactuceae (Asteraceae) (Güzel et al. 2018). It includes annual, biennial or perennial herbs, rarely shrubs with abundant latex. The species of *Lactuca* distributed mainly in warm and temperate geographical regions of the world (van Herwijnen and Manning 2017). However, the continents Asia and Africa are the center of the diversity of the genus. (Doležalová et al., 2002; Lebeda et al. 2004).

The primary gene pool of *L. sativa* L. is represented by a worldwide spread *L. serriola*, further *L. aculeata*, *L. scarioloides*, *L. altaica*, *L. azerbaijancica*, *L. georgica* originating in Asia and *L. dregeana* native to South Africa (Zohary 1991; van Herwijnen and Manning 2017). The secondary gene pool includes *L. saligna*, whereas the tertiary gene pool includes the species that can be crossed with difficulties with *L. sativa*, e.g. *L. virosa* (Doležalová et al. 2002; Jemelková et al. 2018).

Genetic diversity is the raw material permitting species to adapt to environmental changes. The genetic structure of accessions varies from niche to niche along the distribution range of a species (Mondini et al. 2009). So, estimating of genetic diversity is crucial for providing information for domestication, propagation, breeding programs, conservation and use of the germplasm of the plant species (Yu et al. 2001). Biochemical and Molecular markers have been proved as valuable tools to assess and evaluate genetic diversity between and within species, populations and accessions (Yang et al. 2013; Khan et al. 2019). Each marker reveals a specific class of variation, which is dependent on: (1) the fraction of the genome surveyed by that marker, (2) marker distribution throughout the genome and (3) the extent of the DNA target which is analyzed by that marker (Govindaraj et al. 2015; Bhandari et al. 2017).

Isozymes have been used as a reproducible marker in assessing the amount and distribution of genetic variability and systematic relationships within and between *Lactuca* spp. (Kesseli and Michelmore 1986; Dziechciarková et al. 2004). They exhibited that the genetic variability was lower in intra-species compared with inter-species and suggested that the origin of *L. sativa* was polyphyletic. The systematic relationship in *Lactuca* spp. was investigated using isozymes: (1) confirming the genetic closeness between *L. aculeata* and *L. sativa* as they are members of *L. serriola* complex, (2) revealing a wide genetic distance between *L. saligna* and *L. virosa*, and (3) identifying *L. serriola*, *L. saligna*, *L. virosa*, and landrace *L. sativa* as distinct entities (Roux et al. 1985; Kesseli and Michelmore 1986).

The use of molecular markers is also important in assessing the level of genetic diversity and in defining the genetic relationship between and within species, populations and accessions. Randomly Amplified Polymorphic DNA marker (RAPD) is one of the class of DNA markers that has received maximum attention in investigating the genetic variability because it is less expensive, less technical, fast and involves no radioactivity and hybridization. It was used in establishing the differences among lines of apparently closely related accessions in germplasm collections of butterhead and crisphead lettuce (Waycott and Fort 1994). It was also successfully used in identifying between twelve lettuce varieties (Yamamoto et al. 1994). Furthermore, it was used to investigate the genetic variation and interspecific relationship among cultivars of *L. sativa* var. capitata, exhibiting a monophyletic cluster with similarity coefficient values ranging from 0.61 to 0.98 (Yoo and Jang 2003).

Genetic resources collections of *Lactuca* species are poorly characterized because they are made up of large groups of numbered accessions of *Lactuca* that lack descriptive and (or) pedigree (Lebeda et al. 2004 and 2019). So, little information is available on the genetic variation among different accessions of *Lactuca* representing different regions in the world. Therefore, the aim of this research was to use isozymes and DNA markers to evaluate the genetic variability and genetic relationships within and between *Lactuca* species.

Materials And Methods

Plant materials

The experimental material was obtained from the CGN (Centre for Genetic Resources, Wageningen, The Netherlands). It consists of 26 accessions of *Lactuca* spp. (Table 1

Protein extraction, electrophoresis and activity staining

Isozymes crude extracts were prepared by macerating 20 mg young leaves of fifteen days old seedlings with 1 mL of extraction buffer consisted of 0.05M sodium phosphate buffer (pH 7.2), 20% v/v glycerol, 14mM 2-mercaptoethanol and 0.05% v/v triton X-100 (Manchenko 1994). The clear supernatant was applied directly on 7% PAGE at 4°C in a Mini Protean III unit (BioRad, California, USA), under a constant current of 100 mA for 5 to 6hr, until the tracking dye had moved 5 to 7cm from the cathodal end. The gels were subjected to activity staining for Phosphorylase, Catalase, α -Esterase and β -Esterase isozymes following the protocols of Pasteur et al. (1988). Phosphorylase gels were stained in solution formed of 10 mM I_2 mixed with 14 mM KI after incubation in 100 ml solution of 0.1M sodium phosphate buffer (pH 5.1) at 37 °C for 3 to 5 h, developing white bands on a dark blue background. The chromatic or light brown bands appeared at the bottom of the gels were amylase

bands. The gels of catalase were stained by immersing in 1:1 mixture of solutions 2% potassium ferricyanide and 2% ferric chloride after incubation in a solution of 3% H₂O₂ for about 15 min. The gels were then washed and gently agitated for a few minutes in water. Yellow bands of Catalase activity appeared on a blue-green background. The gels of α and β -esterases were incubated at 37°C for 15 min in 100 ml staining solution consisted of 0.05 M phosphate buffer (pH 7.2) containing 1% α or β naphthyl acetate for α and β -esterases respectively and 50 mg Fast Blue RR until brown colored bands appeared. The stained gels were photographed as quickly as possible and stored in 3% acetic acid. At least 5 and generally 10 plants per accession were examined for isozyme patterns

DNA extraction for RAPD analysis

g young leaves of fifteen days old seedlings were ground with a pestle in liquid nitrogen. The ground sample 0.5 was suspended in 1 ml preheated CTAB buffer (1.4 M NaCl, 0.2 % 2-mercaptoethanol, 100 mM Tris-Cl and 20 mM EDTA) at 65°C for one hour. The suspended solution was centrifuge at 1000 rpm and the supernatant was mixed with 0.5 ml of 24:1 chloroform: isomyl, then centrifuge at 14000 rpm (Doyle and Doyle 1990). To precipitate the nucleic acid, the aqueous layer was mixed with ice cold isopropanol, then incubated overnight at -20°C and centrifuged at 14000 rpm. The supernatant was discarded and the pellet was carefully washed twice with cold .70% ethanol, dried at room temperature and re-suspended in 100 μ l of sterile de-ionized distilled water

RAPD amplification

Genomic DNAs of the studied accessions were amplified in 25 μ l reaction mixture, containing 20ng DNA, 0.5 unit Taq polymerase (Sigma-Aldrich, St. Louis, MO, USA), 0.2 mM PCR Nucleotide Mix (Boehringer Mannheim, Tübingen, Germany), 0.5 μ M RAPD primers, 5 μ l amplification buffer, 1.5 μ l of MgCl₂ and 9.75 μ l of distilled H₂O (Williams et al.1990). Amplification was performed for 45 cycles using a Biometra Uno thermal cycler (SPW Industrial, Laguna Hills, CA, USA): One cycle at 95 °C for 3 minutes, then 44 cycles at 92°C for 2minutes, 37 °C for 1minute and 72°C for 2minutes. The reactions were finally run at 72 °C for 10 min and further incubated on ice, at 4°C. The primers were chosen based on their ability to produce reproducible amplification patterns (Table 2). The amplification DNAs were separated by electrophoresis on 2% agarose in 50X Tris-Acetate EDTA buffer consisted of 242g Tris-base, 57.1 ml Glacial acetic acid and 100 ml EDTA (0.5 M pH 8.0). The electrophoresed gels were stained in 0.2 μ g/ml ethidium bromide, then photographed under UV light. DNA ladder (Axygen, Union City, CA, USA) was run side by side with the samples, consisted of the following DNA fragments: 1031, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp

Data analysis

The isozymes and RAPD bands were scored as “0” for presence and “1” for absence for a band at a particular locus in each accessions, which were transformed into a binary character matrix. The binary character matrix of each marker was subjected to multivariate analysis (correspondence analysis and cluster analysis) using the software package “PAST”, Version 4.02, Natural History Museum, University of Oslo, 1999-2020. The cluster .(analyses were performed using the unweighted pair-group method with arithmetic mean (UPGMA

Results

Isozymes analysis

The electrophoretic patterns of the four isozyme systems viz. phosphorylase, catalase, α -Esterase and β -Esterase in 26 accessions of *Lactuca* spp. exhibited 31 alleles (Table 2). 27 alleles were polymorphic and 4 were monomorphic (*ACP-1A*, *ACP-4A*, *CAT-3A* and α *EST-4*) with 87.09% polymorphism (Table 2). Furthermore, the polymorphism in the accessions of *L. sativa* was 74.2%.

The number of alleles ranged from 17 in *L. virosa* CGN05145 from France to 27 in *L. sativax L. serriola* group Oilseed CGN05115 from Egypt with an average 20.8 and the mean number of alleles per locus ranged from 0.55 to 0.87 with an average 0.67 (Table 2). The mean frequency of alleles of the 26 accessions was 0.64. It was higher in cultivated species (0.52) compared to the wild ones (0.48). The lowest allele frequency (0.07) was observed for the allele *CAT-1A* in the accessions of *L. perennis*.

The clustering dendrogram of the isozymes data gave two main groups (G1 and G2) at genetic distances 2.25 (Figure 1). The accessions of *L. sativa* were distributed in the two groups (Figure 1). G1 included *L. serriola* group Oilseed lettuce, *L. serriola*, *L. sativa x L. serriola* group Oilseed and six accessions of *L. sativa* (Figure 1). G2 contained accessions of *L. dregeana* with the rest of the accessions of *L. sativa*. At genetic distances 1.9, *Lactuca* accessions were separated into five clusters and two singletons (*L. sativax L. serriola* group Oilseed (CGN05115) and *L. perennis* CGN13299). Cluster one (C1) contained the accessions of *L. indica* and *L. perennis* CGN09321, whereas C2 included *L. sativa* (group Butterhead lettuce CGN04888, group Crisp lettuce CGN05048, group Latin lettuce CGN04566, group Latin lettuce CGN04557, group Cos lettuce CGN04744 and group Stalk lettuce CGN11387), *L. serriola* group Oilseed lettuce CGN04770, *L. serriola* CGN16210 and *L. saligna* CGN13330. *L. saligna* CGN13327, *L. saligna* CGN10883 and the accessions of *L. viminea* were collected in C3. The accessions of *L. virosa* and *L. dregeana* were separated in C4. *L. sativa* group Butterhead lettuce CGN04706, *L. sativa* group Latin lettuce CGN05835, *L. sativa* group Cos lettuce CGN04744 and *L. sativa* group Cutting lettuce CGN10956 were grouped in C5.

The corresponding analysis of the *Lactuca* accessions based on their isozymes characteristics is presented in Figure 2. The accessions in the top right quadrant (*L. sativa* group Butterhead lettuce CGN04888, *L. sativa* group Crisp lettuce CGN05048, *L. sativa* group Latin lettuce CGN04566, *L. sativa* group Latin lettuce CGN04557, *L. sativa* group Cos lettuce CGN04744, *L. sativa* group Stalk lettuce CGN04546, *L. serriola* group Oilseed lettuce CGN04770, *L. serriola* CGN16210, *L. sativax L. serriola* group Oilseed CGN05115, *L. perennis* CGN13299) were closely associated by the alleles, namely *CAT-1A*, *CAT-2A*, *CAT-4A*, α *EST-1A*, α *EST-2A*, α *EST-3B* and β *EST-3A* (Figure 2). The left top quadrant consisted of the accessions (*L. sativa* group Butterhead lettuce CGN04706, *L. sativa* group Latin lettuce CGN05835, *L. sativa* group Cutting lettuce CGN10956 and the accessions of *L. viminea*) with the alleles *ACP-2C*, *ACP-3A*, *CAT-4C*, α *EST-2C*, β *EST-1A*, β *EST-2A* and β *EST-3C*. The right bottom quadrant comprised the accessions *L. saligna* (CGN13330, *L. indica* CGN14312, *L. indica* CGN13392, *L. perennis* CGN09321) that were closely associated with *ACP-2B*, *ACP-3B*, *CAT-4B*, α *EST-2B*, β *EST-2B*, β *EST-3B*. The accessions (*L. saligna* CGN13327, *L. saligna* CGN10883, *L. virosa* CGN05332, *L. virosa* CGN05145, *L. dregeana* GN04790, *L. dregeana* CGN05805) in the bottom left were also closely related in terms of *CAT-2B*, α *EST-1B*, α *EST-3A*, β *EST-1B*, β *EST-4B*. The distance between the accessions on the score plot

is directly associated with the degree of divergence or similarity among them in respect to their isozymes characters.

RAPD analysis

Among 18 random primers tested in this study, 5 primers generated reproducible bands. A total of 186 polymorphic bands were identified using the five primers. Maximum and minimum percentages of polymorphic bands were observed by primers OPA3 (46 fragments) and OPA1 (31 fragments) respectively. The average percentage of polymorphic bands was 100 % (Table 3). The average size of DNA fragments ranged between 33bp and 600bp (Figure 3). The fragment with DNA size of: 33 bp is unique marker for *L. sativa* x *L. serriola* group Oilseed CGN051115, 500 bp DNA fragment for *L. indica* CGN13392 (Primer 1 OPA09); 200 bp DNA fragment for *L. serriola* group Oilseed lettuce CGN04770, 500 bp DNA fragment for *L. serriola* group Oilseed lettuce CGN04770 (Primer 2 OPA14), 200 bp DNA DNA fragment for *L. serriola* group Oilseed lettuce CGN04770, 350 bp DNA fragment for *L. saligna* CGN13330, 47 bp DNA fragments for *L. saligna* CGN10883 (Primer 3 OPZ12).

The clustering dendrogram constructed based on RAPD results showed seven clusters (C1-C7) (Figure 4). The first and second clusters (C1 and C2) included *L. serriola* group Oilseed lettuce CGN04770 and all the accessions of *L. sativa* except *L. sativa* group Butterhead CGN04706 from which was separated as singleton at far genetic distance. The wild species *L. serriola* CGN16210 and *L. sativa* x *L. serriola* group Oilseed CGN051115 were clustered in C7 which is connected with other accessions at genetic distance 7.5. The accessions of *L. viminea*, *L. dregeana* and *L. perennis* were included in C5. The accessions of *Lactuca saligna* (C3 and C6), *L. virosa* (C3 and C4) and *L. indica* (C4 and C5) were distributed in two clusters. It can be noticed that two accessions of *Lactuca saligna* (*Lactuca saligna* CGN13327 from Greece and *Lactuca saligna* CGN10883 from Portugal) were collected with *Lactuca serriola* and *Lactuca sativa* x *Lactuca serriola* group Oilseed and the third accession (*Lactuca saligna* CGN13330 from Turkey) was clustered with *Lactuca virosa* and *Lactuca indica*.

Discussion

Estimating the genetic diversity between and within accessions of crop species and its wild relatives assist in decision-making to select the best accessions for selection of parents for hybridization and is crucial for providing information for domestication and propagation (Yu et al. 2001; Khan et al. 2019). It is also enhancing the understanding of the plant germplasm for germplasm management and potential users, and helping in producing new varieties best adapted to regional environmental conditions (El-Esawi, et al. 2017; van Herwijnen and Manning 2017).

In the present study, the estimation of genetic diversity with isozyme markers showed high polymorphism between the examined accessions (87.09%) which was attributed to outcrossing of the majority of the species and infrequent interspecific hybridization (Jemelková, et al. 2018). It also showed that the polymorphism between *L. sativa* accessions (74.2%) was not with same magnitude of the polymorphism between the wild species, reflecting the impact of domestication (successive rounds of selection) on reducing the genetic diversity of the domesticated species, leaving them with less allelic richness than their wild progenitors and other crop wild relatives (Abbo et al. 2014; Dempewolf et al. 2017).

The highest number of alleles in the wild species (31) compared with the cultivated species *L. sativa* (27) reflected a reduction in the richness of alleles due to domestication which was reported to modify agronomic phenotypes and genetic signature of the domesticated species, resulted in the reduction in genetic diversity to meet the demand for high crop productivity and crop uniformity in the field and the marketplace (Dempewolf et al. 2017; Zhang et al. 2017). The variation between cultivated and wild species was not only limited to the number of alleles, but also it extended to the mean frequency of alleles which was found to be higher in the cultivated species (0.52) comparing with the wild ones (0.48). This might be attributed to the absence of selection pressure on the wild species, as the existing variations in these species are natural (Das 2011). The allele with low mean allele frequency (0.07) or what was known as rare alleles was observed only for the allele *CAT-1A* in *L. perennis*. The presence of this allele could be due to deleterious mutations or may be due to evolutionary relics (Sammour et al. 2019). The detection of rare allele in combination with high allelic frequency of other loci leads to the conclusion that the studied accessions had wide genetic differentiation.

The separation of *L. dregeana* with *L. virosain* the same cluster in isozymes and RAPD cluster analyses confirmed their closely relatedness as they belong to the same section *Lactuca* (Lebeda and Astley 1999), having one pair of satellites (El-Esawi and Sammour 2014) and similar morphological characters (De Candolle, 1838). Although, Koopman et al. (1998), Koopman et al. (2001) and Wei et al. (2015) confirmed the very close relationship between *L. serriola* and *L. dregeana* and considered the two taxa to be conspecific based on AFLP fingerprints, nuclear and plastid DNA sequence comparison, isozymes and RAPD results of the present research recognized differences between them adequate to maintain them as distinct species, confirming the previous morphological, karyological, historical occurrence, and ecological studies (Zohary 1991; El-Esawi and Sammour 2014; van Herwijnen and Manning 2017).

The distribution of the accessions of *L. sativa* in more than one cluster in the isozymes and RAPD dendrograms confirmed the polyphyletic origin of *L. sativa*. Furthermore, the clustering of some of the accessions of *L. sativa* with *L. serriola* group *Oilseed lettuce* based on isozymes data and the clustering of the rest of the accessions with *L. dregeana* confirmed that *L. serriola* and *L. dregeana* were members of the primary gene pool of *L. sativa* (Zohary 1991) and was consistent with the work of Kesselli and Michelmore (1986) who also suggested that domestication of *L. sativa* may be due to the repeated domestication from wild progenitors or may be due to the use of interspecific hybridizations in breeding programmes to introduce characters of interest into cultivated lettuce. However, the prevailing of the allele "B" in the wild species and alleles "A" and "C" in the cultivated species and its progenitors, as it has been shown in the corresponding analysis, indicating that the cultivated species was firstly domesticated from wild progenitors and later breeders used interspecific hybridizations to introduce characters of interest into cultivated lettuce. The prevailing of the allele "B" in wild species and alleles "A" and "C" in cultivated ones also suggested that: (1) allele "B" might be the primitive form of studied alleles that enable the wild species to resist the environmental stresses which prevails in their habitats, and (2) alleles "A" and "C" could be derived forms that evolved as a result of domestication.

The separation of the accessions of *L. saligna* in two clusters in the cluster analysis of the isozymes data was consistent with the works of Cole et al. (1991) and Esawi et al. (2017). The heterogeneity of this species attributed to the difference in the number and types of alleles in the studied accessions. The accessions *Lactuca saligna* CGN13327 from Greece and *Lactuca saligna* CGN10883 from Portugal did not have the allele "A" of the loci *ACP-2*, *CAT-2*, *CAT-4*, α *EST-2*, β *EST-3*, β *EST-4* which characterize the cultivated species, whereas the

accession *Lactuca saligna* CGN13330 from Turkey had these alleles. This led to infer that the accession from Turkey might be subjected to a natural mutation or a sort of unintended domestication. Although Güzel et al. (2018) observed several samples in the field and herbaria belonging to *L. viminea* that easily fell into two distinct subspecies according to their habits and morphological traits, the clustering dendrograms of isozymes and RAPD analyses separated the accessions of *L. viminea* in one cluster which was inconsistent with their observation. The variation observed by Güzel et al. (2018) could be attributed to ecogeographical conditions in which the accessions of *L. viminea* were originated; the conditions that cause a significant variation in the phenotypic characters. The clustering dendrogram based on RAPD data indicated a close genetic relationship between *L. sativa*, *L. serriola*, *L. saligna* and *L. virosa* which was consistent with the phylogenetic trees based on chloroplast DNA sequence comparison (Wei et al. 2017), chromosomal studies (Matoba et al. 2007), nrITS1 and AFLP fingerprints (Koopman et al. 1998; Koopman et al. 2001). So, *L. serriola*, *L. saligna* and *L. virosa* can be considered important resources for *L. sativa* breeding.

Conclusion

The polymorphism in the studied accessions were very high either in isozymes or RAPD analyses, exhibiting high genetic variability within and among *Lactuca* species. The polymorphism and the number of alleles were lower in the cultivated species compared to the wild species, reflecting a reduction in the richness of alleles in the cultivated species due to domestication that caused a reduction in genetic diversity of the cultivated species comparing with the wild ones. The separately multivariate analyses of the isozymes and RAPD data confirmed the polyphyletic origin of *L. sativa*. They also collected the accessions of *L. viminea* in one cluster revealing the homogeneity of this species and ruling out the previous study that showed that *L. viminea* has two distinct subspecies. The corresponding analysis associated the accessions of the wild species based on the alleles "B" of the studied isozymes, the form that might enable the wild species to resist to the environmental stresses that prevails in their habitats; and the cultivated species on alleles "A" and "C". This suggested that allele "B" might be the primitive form of the alleles of the loci of the assessed isozymes, and "A" and "C" could be the derived forms. The separation of the accessions of *L. saligna* in two clusters was due to the variation in the number of alleles, particularly "A" form, suggesting that some accessions could be wild and the other might be subjected to natural mutation or unintended domestication. Accessions of *L. sativa* x *L. serriola* group Oilseed, *L. indicia*, *L. saligna* were characterized with unique DNA fragments which can be used as markers for identifying these accessions. The presence of the great genetic variation between cultivated lettuce and its wild relatives opened the path for the improvement of the cultivated species. The association between specific forms of alleles and cultivated and wild species still needed deep insights to be manipulated for lettuce improvement. The considerable genetic variation in the accessions of *Lactuca saligna*, *Lactuca virosa* and *Lactuca indica* open the door for more detailed studies on big number of accessions cover the distribution range of these species using morphological, biochemical and molecular markers.

Declarations

Acknowledgment

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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Tables

Tables not available with this version.

Figures

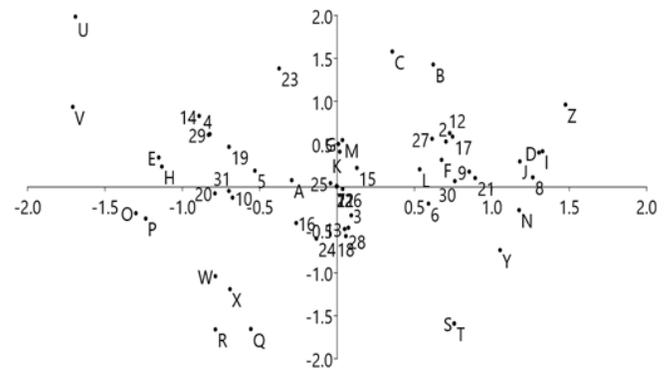
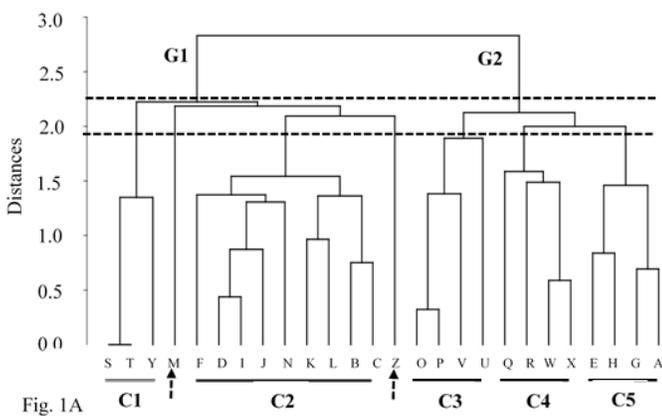


Figure 1

A. UPGMA clustering of 26 accessions of *Lactuca* species based on isozymes data. B. Corresponding analysis of 26 accessions of *Lactuca* spp. based on their isozymes characteristics explained by the first and second principal components.

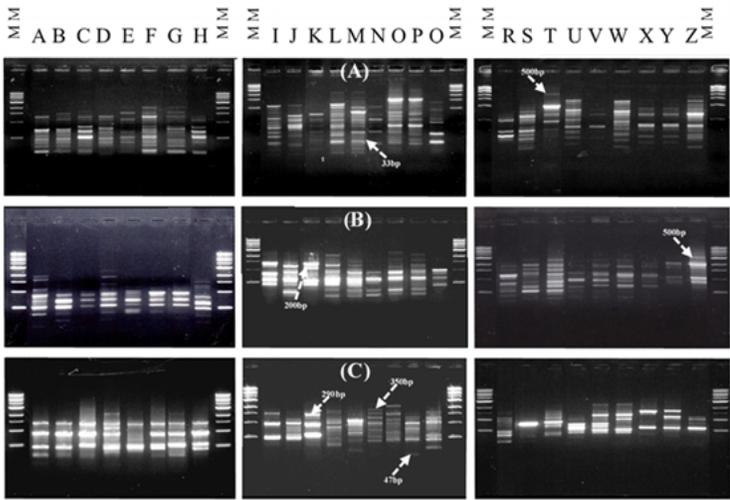


Fig. 2A

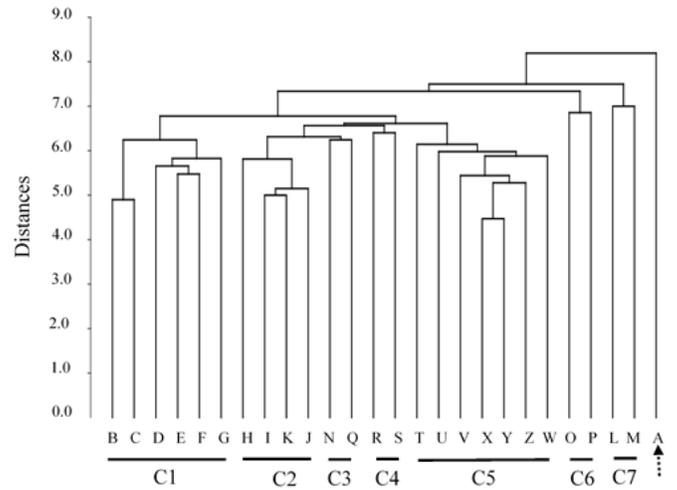


Fig. 2B

Figure 2

A. RAPD profiles of 26 accessions of *Lactuca* species produced by primer OPA09 (A), primer OPA14 (B), primer OPZ12 (C). The arrows indicates accession specific marker. B. UPGMA dendrogram of 26 accessions of *Lactuca* species based on RAPD marker data.

Figure 3

Figure 3 not provided with this version

Figure 4

Figure 4 not provided with this version